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Serum levels of matrix metalloproteinase-10 are associated with the severity of atherosclerosis in patients with chronic kidney disease

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Cardiovascular disease is the leading cause of mortality in chronic kidney disease (CKD). As matrix metalloproteinases have a major role in atherosclerosis, we hypothesized that alterations in metalloproteinases-8, -10 and their tissue inhibitor-1 can be associated with the severity of atherosclerosis in patients with kidney disease. This was evaluated in a cross-sectional, observational study of 111 patients with stages I–V kidney disease, 217 patients on dialysis and 50 healthy controls. The severity of atherosclerosis was estimated with the atherosclerosis score (AS), combining the results of ankle–brachial index and carotid ultrasound. Serum levels of the two metalloproteinases and tissue inhibitor-1 were measured by enzyme-linked immunosorbent assay and were significantly increased in patients with kidney disease compared with the healthy controls, and higher in patients on dialysis than in earlier stages of CKD. The severity of the AS was also more prevalent in the dialysis group, in which serum levels of both metalloproteinases and tissue inhibitor-1 were significantly higher. After multivariate analysis, metalloproteinase-10, dialysis, C-reactive protein, age, and male gender were associated with increased risk of atherosclerosis. Thus, patients with CKD exhibit elevated levels of circulating metalloproteinase-10, and this was independently associated with the severity of atherosclerosis and may represent a new biomarker of atherosclerotic diseases.

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Cardiovascular diseases (CVD) are the leading cause of death in patients with chronic kidney disease (CKD),[1,2] and cardiovascular risk is higher in such patients than in the general population even at early stages of CKD.[3–5] Several factors have been involved in the high incidence of CVD in CKD patients. The burden of traditional risk factors (high blood pressure, diabetes mellitus) is higher in CKD than in the general population[6] and it is associated with non-traditional variables such as anemia,[7] oxidative stress, endothelial dysfunction,[8] and an increased prevalence of inflammation as kidney function declines.[9,10] Moreover, the presence and severity of atherosclerosis is significantly higher in CKD-affected patients at any group of age compared with control subjects with normal kidney function.[11]

The balance between matrix metalloproteinases (MMPs) and their specific tissue inhibitors (tissue inhibitor of metalloproteinases, TIMPs) regulates the proteolysis of the vascular extracellular matrix and is crucial for atherosclerosis and plaque destabilization.[12] The proteinase activities exerted by MMPs have been implicated in some of the biological processes associated with atherosclerosis and its ischemic clinical manifestations, such as myocardial infarction and stroke, and circulating MMP levels have been associated with subclinical atherosclerosis and increased cardiovascular risk (reviewed by Bäck *et al.*[13]). However, the roles of MMPs and TIMP in the pathogenesis of atherosclerosis in CKD are poorly understood. Among different MMPs, we have paid special attention to MMP-8 and MMP-10. Serum MMP-8 concentration is elevated in subclinical atherosclerosis and is associated with worse cardiovascular outcomes.[14] Recent studies from our group have also shown that different inflammatory/prothrombotic stimuli induce a significant expression and secretion of MMP-10 by human endothelial cells, and this is also found in human atherosclerosis.[15–17]

We hypothesized that CKD is associated with alterations of MMPs and TIMPs that may be related to the severity of atherosclerosis in this clinical setting. Therefore, we measured

Table 1 | Selected characteristics of the study population (*N*=378)

	Controls, *N*=50	CKD stages I–III, *N*=43	CKD stages IV–V, *N*=68	Dialysis, *N*=217	*P*-value
Demographic data					
Age, years (s.d.)	64.9 (3)	59.6 (11)†	69.2 (12)†	64.7 (12)†	0.001
Sex, female, *N* (%)	30 (60)	29 (67.4)	38 (55.9)	134 (61.6)	0.66
Diabetes mellitus, *N* (%)	8 (16)	34 (79.1)	43 (63.2)	146 (67.6)	0.02
High blood pressure, *N* (%)	32 (64)	37 (86)	63 (92)	130 (59)	0.001
Lipid-lowering treatment, *N* (%)	10 (20)	22 (51.2)	35 (51.5)	96 (44.2)	0.002
Smoking, *N* (%)	2 (4.2)	7 (16.7)	11 (18.3)	38 (17.6)	<0.001
Previous CV disease, *N* (%)	3 (6)	5 (11.6)	17 (25)	87 (40.3)	<0.001
Clinical and laboratory data					
BMI, kg/m^2	28.3 (4.3)*	29.4 (4.2)	28.6 (5.5)	25.6 (4.7)†	<0.001
Systolic blood pressure, mm Hg	136 (22)	137 (16)	140 (24)	136 (24)	0.67
Diastolic blood pressure, mm Hg	82 (8)*	78 (10)	75 (10)†	71 (12)†	<0.001
Glucose, mg/dl	108 (37)	112 (33)	108 (40)	116 (51)	0.54
Total cholesterol, mg/dl	221 (39)*	184 (27)†	171 (31)†	157 (35)†	<0.001
HDL cholesterol, mg/dl	52 (12)	56 (17)†	52 (13)	47 (17)†	0.002
LDL cholesterol, mg/dl	147 (37)*	102 (25)†	91 (25)†	81 (30)†	<0.001
Triglycerides, mg/dl	112 (53)*	138 (66)	142 (79)	148 (98)†	0.06
C-reactive protein, mg/l	0.45 (0.58)*	5.18 (7.6)†	6.07 (9.1)†	15.79 (28)†	<0.001
Calcium, mg/dl	NA	9.4 (0.3)†	9.1 (0.6)†	8.8 (0.6)†	<0.001
Phosphorus, mg/dl	NA	3.3 (0.5)†	3.9 (0.6)†	4.7 (1.4)†	<0.001
Atherosclerosis burden					
Carotid IMT, mm	0.74 (0.16)*	0.81 (0.14)	0.80 (0.16)	0.83 (0.16)†	0.008
Carotid plaque, *N* (%)	13 (28)	20 (46.6)	47 (69.1)	171 (79.1)	<0.001
Ankle–brachial Index	NA	0.95 (0.1)	0.90 (0.2)	1.01 (0.4)	0.06
Atherosclerosis score (AS)					<0.001
AS 0/1, *N* (%)	NA	22 (51.2)	18 (26.5)	33 (15.3)	
AS 2/3, *N* (%)	NA	21 (48.8)	50 (73.5)	183 (84.7)	

Abbreviations: BMI, body mass index; CKD, chronic kidney disease; CV, cardiovascular; HDL, high-density lipoprotein; IMT, intima-media thickness; LDL, low-density lipoprotein; NA, not assessed.
*Indicates $P<0.005$ in the *post hoc* analyses of Bonferroni when compared controls vs CKD stages I–III, IV–V, and dialysis.
†Indicates $P<0.005$ in the *post hoc* analyses of Bonferroni when compared dialysis vs CKD stages I–III and IV–V.

the circulating concentrations of MMP-8, MMP-10, and TIMP-1 in patients at different stages of CKD, and analyzed the impact of MMPs in the atherosclerotic burden of these patients.

RESULTS

General characteristics of the study population

We included 328 patients (61.2% females, 65 (13) years old) and 50 age- and sex-matched controls (60% females, 64.9 (3) years old). Demographic, laboratory, and atherosclerosis assessment results are displayed in Table 1. We found significant differences when laboratory values (not demographic and clinical data) were analyzed separately by sex, as males presented with lower total, high-density lipoprotein, low-density lipoprotein, and triglyceride concentration than females, at every stage of CKD. This difference was not observed in control subjects. Patients at stages IV–V of CKD were significantly older than patients at earlier stages, patients on dialysis, and controls. There were no significant differences in gender distribution, although the prevalence of diabetes mellitus, hypercholesterolemia, high blood pressure, smoking status, and previous CVDs were significantly higher in the group of CKD patients than in controls. As previously described in the literature, patients on dialysis had lower values of body mass index, diastolic blood pressure, total cholesterol, high-density lipoprotein, and low-density lipoprotein cholesterol (*P*-values overall <0.05, Table 1) when compared with both groups of patients at earlier stages of CKD and controls. Conversely, the concentration of C-reactive protein was significantly higher in the dialysis group than in the other groups.

CKD patients presented with significantly higher carotid intima-media thickness (cIMT) values and higher prevalence of carotid plaques than controls, although total and low-density lipoprotein cholesterol concentrations were significantly lower. Furthermore, the prevalence of carotid atheromatous plaques was significantly higher in later stages of CKD, reaching 79.1% of patients on dialysis ($P<0.001$, Table 1). Similarly, more severe atherosclerosis score (AS) was far more prevalent in stages IV–V and dialysis than in stages I–III (73.5, 84.7, and 48.8%, respectively, $P<0.001$).

MMPs at different stages of CKD

The concentrations of MMP-8, -10, and TIMP-1 according to the stage of CKD are displayed in Table 2. CKD patients exhibited much higher ($P<0.001$) concentration of MMP-8, -10, and TIMP-1 than controls. MMP-10 concentrations were higher ($P=0.004$) in those patients in the group of dialysis

Table 2 | Serum MMPs concentration according to stages of CKD

	Controls, *N*=50	CKD stages I–III, *N*=43	CKD stages IV–V, *N*=68	Dialysis, *N*=217	*P*-value
MMP-8, ng/ml†	11.21 (12)	30.75 (26)*	26.70 (21)*	43.03 (41)*	<0.001
MMP-10, pg/ml	555 (173)	1513 (1096)*	1814 (853)*	2099 (1138)*	<0.001
TIMP-1, ng/ml†	245.25 (90)	193.38 (75)	243.59 (62)	305.22 (120)*	<0.001

Abbreviations: CKD, chronic kidney disease; MMP, matrix metalloproteinase; TIMP-1, tissue inhibitor of metalloproteinases type-1.
*Indicates $P<0.005$ in the *post hoc* analyses of Bonferroni.
†MMP-8 was measured in 29 and TIMP-1 in 44 out of 50 controls.

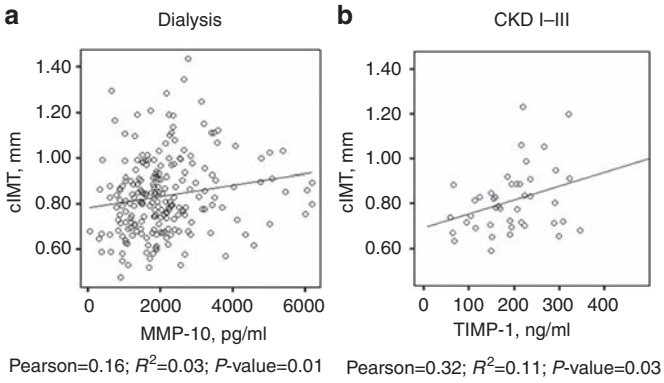

Figure 1 | Association between carotid intima-media thickness (cIMT) and proteolytic markers in the study population. Significant correlations between cIMT and concentration of matrix metalloproteinase (MMP)-10 (**a**) and tissue inhibitor of metalloproteinases type 1 (TIMP-1) (**b**) in patients under dialysis and chronic kidney disease (CKD) I–III, respectively.

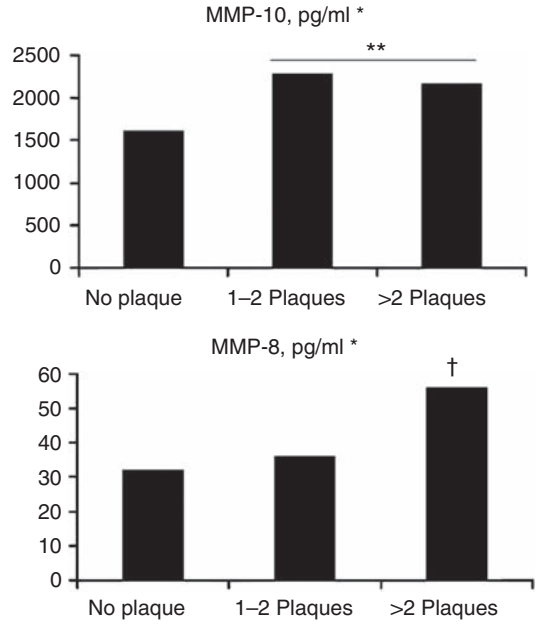

Figure 2 | Distribution of matrix metalloproteinase (MMP)-10 and MMP-8 in patients under dialysis according to the number of carotid plaques. *Analysis of variance, *P*-value = 0.003 for MMP-10 and 0.001 for MMP-8. **Post hoc* analyses of Bonferroni; $P<0.05$ between no plaque and the other groups. †*Post hoc* analyses of Bonferroni; $P<0.05$ between more than two plaques and the other groups.

than those on stages I–III (Bonferroni *post hoc* analyses). Similarly, MMP-8 and TIMP-1 were significantly increased in the group of dialysis when compared with the other groups (Bonferroni *post hoc* analyses, $P<0.005$, Table 2).

MMPs and AS in CKD patients

We diagnosed 254 (77%) CKD patients with either moderate or severe atherosclerosis (AS 2 or 3). CKD patients with AS 2/3 were significantly ($P<0.001$) older (67–11)) than those with AS 0/1 (56 (15)). We did not find significant differences between groups (AS 0/1 vs AS 2/3) pertaining to diabetes mellitus, high blood pressure, treatment with lipid-lowering agents, and smoking. Similarly, there were no differences in body mass index and systolic blood pressure values. However, patients diagnosed of moderate or severe atherosclerosis presented with higher concentration of C-reactive protein than those patients with no or mild atherosclerosis (14.4 (26) vs 5.6 (6), mg/l, $P=0.006$). Conversely, lipid values (total, high-density lipoprotein, and low-density lipoprotein cholesterol) were significantly lower in the group with moderate or severe atherosclerosis (data not shown).

We then analyzed the concentration of MMPs according to the AS, and we found a significant ($P<0.05$) increase in the concentrations of MMP-8, -10, and TIMP-1 in CKD patients with moderate or severe atherosclerosis when compared with those with no or mild atherosclerosis. We then analyzed markers of atherosclerosis burden (cIMT (Figure 1) and number of carotid plaques (Figure 2)) and their relationship with MMPs/TIMP-1 according to the different stages of CKD. We did not find significant correlations between cIMT and MMPs/TIMP-1 at the different stages of CKD, with the exception displayed in Figure 1. In the group of patients under dialysis, the concentration of MMP-10 was positively (Pearson coefficient 0.16) and significantly ($P=0.01$) associated with cIMT. Similarly, in patients at CKD stages I–III, the concentration of TIMP-1 was positively (Pearson coefficient 0.32) and significantly ($P=0.03$) related to cIMT. As displayed in the Figure 2, the severity of carotid atherosclerosis (defined by the number of carotid plaques) was related to significantly higher concentrations of MMP-10 and -8. Those patients under dialysis with either 1–2 or multiple carotid plaques presented with significantly higher concentrations of MMP-10 and -8, than patients without plaques. These results were not reproduced in patients at different stages of CKD and controls.

We also analyzed the impact of MMPs on the severity of atherosclerosis in the multivariate analyses. Independent variables were selected from the univariate analyses ($P<0.10$)

Table 3 | Multivariate analysis of atherosclerosis score in patients with CKD

	Odds ratio	Confidence interval (95%)	*P*-value
CKD stage, dialysis vs I–III	1.57	1.19–2.07	0.001
Age, years	1.06	1.04–1.09	<0.001
Sex, female vs male	0.42	0.22–0.79	0.008
Previous CV disease, yes vs no	1.25	0.53–2.95	0.60
Diabetes mellitus, yes vs no	1.32	0.63–2.75	0.46
Lipid-lowering treatment, yes vs no	1.41	0.75–2.67	0.28
Score risk	0.84	0.30–2.33	0.74
C-reactive protein, mg/l	1.03	0.99–1.08	0.05
MMP-10, upper tertile vs first tertile	1.57	1.06–2.32	0.02
MMP-8, upper tertile vs first tertile	1.15	0.77–1.73	0.47
TIMP-1, upper tertile vs first tertile	1.00	0.65–1.54	0.97

Abbreviations: CKD, chronic kidney disease; CV, cardiovascular; MMP, matrix metalloproteinase; TIMP-1, tissue inhibitor of metalloproteinases type-1.

and included: age, sex, CKD stage, C-reactive protein, cardiovascular risk measured by score, previous CVDs, diagnoses of diabetes mellitus, lipid-lowering therapy, and the concentration of MMPs and TIMP-1. As the dependent variable we selected the AS, and the significant results are listed in Table 3. Being on dialysis, older age, male, and the higher concentration of MMP-10 significantly increased the risk of moderate or severe atherosclerosis (Table 3). Interestingly, we did not find significant associations with the previous diagnoses of CVD or the concentration of MMP-8 and TIMP-1.

DISCUSSION

Turnover of extracellular matrix proteins, crucial for atherosclerotic plaque development and rupture, is largely achieved through the action of MMPs, which represent a major class of matrix-degrading proteinases. We report herein that serum MMP-8, 10, and TIMP-1 are abnormally increased in CKD patients, in particular in those in dialysis. In addition, we found that MMP-10 is associated with the severity of atherosclerosis in CKD patients, thus indicating that MMP-10 may represent a new biomarker of atherosclerosis in this clinical setting.

It is well accepted that MMPs are key factors in atherosclerosis, being implicated in intimal thickening and in the subsequent plaque rupture.[12,13,18] However, serum concentrations of MMP-8 and -10 have not been previously determined in CKD patients. MMP-8 levels correlate with the presence and severity of coronary artery disease,[14] and are associated with carotid plaque instability after stroke and subclinical atherosclerosis.[19] Recently, our group reported that MMP-10 is expressed in atherosclerotic plaques, being almost undetectable in healthy arteries, and that endothelial MMP-10 expression can be induced by inflammatory stimuli.[15–17] Similarly, systemic levels of MMP-10 correlate with inflammatory markers and are associated with atherosclerosis.[16,17]

In this study, we found that the concentrations of MMP-8, -10, and TIMP-1 were significantly increased in dialysis patients compared with those on stages I–III. Interestingly, when analyzing the concentration of MMPs according to the severity of atherosclerosis, as assessed by the AS, cIMT, and number of carotid plaques, we found that concentrations of MMP-8, -10, and TIMP-1 were significantly higher in the group of patients with more severe atherosclerosis when compared with those with no or mild atherosclerosis. Furthermore, in those subjects on dialysis a positive and significant correlation between cIMT and MMP-10 was found. Similarly, the concentration of TIMP-1 was significantly correlated with cIMT in patients at stages of CKD I–III. In the multivariate analysis, we found that being on dialysis, age, C-reactive protein, and the concentration of MMP-10 were significantly associated with the risk of severe atherosclerosis. Taken together, our results suggest that circulating MMP-10 concentration is an independent risk factor for atherosclerosis in CKD patients.

Our study has several limitations. This is a cross-sectional, observational study, and therefore we do not have data pertaining to the prognostic value of MMPs on the incidence of CVD. We have to keep in mind, however, that we used a validated score for atherosclerosis assessment, which has been previously tested as a prognostic tool to better predict coronary heart disease.[20] Controls ($n = 50$) have been obtained from a previous study, including 400 apparently healthy subjects, and are age and sex matched with patients under study. Furthermore, our control subjects are representative of the general healthy population in Spain, as similar results, in terms of cardiovascular risk factors, have been obtained from two population-based cross-sectional surveys.[21,22] Despite the intrinsic limitations of a case–control study, we are confident that controls data are representative of healthy Spanish population, and then the conclusions of the study remained valid.

Another limitation is the heterogeneity of the population under study, with a predominance of those patients on dialysis. This might be the reason why the strongest association between atherosclerosis burden and MMPs has been found in the group under dialysis, with the exception of TIMP-1 in CKD stages I–III. Whether MMP-10 concentration is significantly influenced by dialysis and has nothing related to atherosclerosis development remains unknown, and further studies should be performed.

We do think, however, that these limitations do not affect the conclusion of the study, as the results of the multivariate analyses are very conclusive, regarding the association of MMP-10 to the severity of atherosclerosis. This observational study should guide future research in experimental models, in order to elucidate involved mechanisms and therapeutic targets.

We did not perform mechanistic studies to ascertain the source of the excess of MMPs in our patients. In addition to the inflammation caused by subclinical atherosclerosis and renal failure, there are various potential causes of MMP elevation as found in CKD patients. For example, the host response to the insult of pathogens results in increased

MMPs.[23–25] MMPs could also be involved in vascular calcification, which occurs pathologically in diabetes and CKD, as well as during the normal ageing process.[26,27] Vascular calcification in patients with advanced stages of CKD is associated with significant shorter survival, and its relationship with MMPs will be a focus of research in the future.

In conclusion, for the first time, we found that serum MMP-8, -10, and TIMP-1 are abnormally increased in CKD patients, especially in those on dialysis, and that MMP-10 is associated with more severe atherosclerosis. Albeit preliminary, the reported findings set the stage for further studies aimed at determining whether serum MMP-10 may be of prognostic value in assessing the risk of atherosclerotic events in CKD patients.

MATERIALS AND METHODS

Design

This is a cross-sectional, observational study, performed in collaboration between the University-based Hospital Arnau de Vilanova and the Center for Applied Medical Research of the University of Navarra. The protocol has been reviewed and approved by the Institutional Ethical Review Board of the Hospital, and each participant has signed the informed consent. Inclusion period was set at 6 months, during which we consecutively invited patients to participate.

Study population

We included both modalities of dialysis ($N=217$), hemodialysis ($N=191$), and peritoneal dialysis ($N=26$). We did not exclude participants according to age, previous CVDs, or concomitant medical conditions, with the aim to have a representative population on dialysis.

We similarly recruited patients at earlier stages of CKD, I–IV, and V (No D), defined as recommended by current guidelines,[28] from the specialized nephrology outpatient clinic.

We also included healthy subjects as controls, as described in a previously published article.[29] Briefly, we identified 50 age- and sex-matched subjects, with normal kidney function (according to creatinine (mean (s.d.): 0.83 (0.18) mg/dl) and AC-MDRD-4 estimation), who attended the outpatient service of the Department of Internal Medicine at the University Clinic of Navarra for a general check-up. Carotid ultrasound was performed using the same standard operational procedure as in patients (see below), and the technician was blind to demographic and clinical characteristics of participants. Ankle–brachial index (ABI) was not assessed in this subset of participants.

Procedures and variables

Demographic, clinical, and laboratory variables were recorded in a unitary database. Highly specialized personnel, blinded to participant-related information, performed the procedures, which consisted in a standardized protocol for the non-invasive assessment of atherosclerosis: carotid, abdominal ultrasound, and ABI, as recently published.[11]

1. *Carotid ultrasound*: to measure cIMT and to identify carotid plaques. We used a MicroMaxx, SonoSite (Bothell, WA, USA) with a linear transducer HFL38/13-6 MHz. Briefly, the protocol consists in:
   - Cross-sectional loop of the region of interest (common carotid, bulb, and internal) to identify atheroma plaques.
   - *Longitudinal images*: as it is defined in the consensus,[30] we analyzed the far wall of the common carotid artery in the last centimeter, bulb section and finally internal carotid artery in the first centimeter. The measurement of the cIMT is performed using the semi-automated, Food and Drug Administration-approved software, SonoCalc IMT (Bothell, WA, USA).
   - *Pulsed-Doppler*: we use the color and pulsed-Doppler once an atheromatous plaque is identified (significant stenosis considered when systolic velocity peak >125 cm/s is reached).
2. Abdominal ultrasound: to measure the presence of aortic aneurysm, using a convex transducer C60 (Bothell, WA, USA).
3. *Measurement of ABI*: we used a vascular Doppler MD2 Hungleight (Bothell, WA, USA) with an 8 MHz transducer. We used maximum brachial systolic pressures and recorded ABI as the lowest value obtained at each territory.

We set an AS, according to previous literature,[11,20] as follows:

1. *No atherosclerosis (AS 0)*: ABI >0.9 and cIMT inferior to the cut-off value representing the 80% reference interval adjusted by age and sex. Reference interval values have been obtained from previously published observational studies using the same ultrasound procedures.[31,32]
2. *Mild atherosclerosis (AS 1)*: an ABI 0.7–0.9 and/or cIMT superior to the cut-off value of the 80% reference interval.
3. *Moderate atherosclerosis (AS 2)*: the presence of a carotid plaque without significant stenosis (<125 cm/s) and ABI $\geqslant 0.7$.
4. *Severe atherosclerosis (AS 3)*: an ABI <0.7 and/or the presence of a carotid plaque with significant stenosis (>125 cm/s).

We did not include the results of abdominal scan in the AS, as we did not find any patient with abdominal aortic aneurysms.

For statistical purposes and clear display results, we have grouped patients with AS 0 and AS 1 in one group (no and mild atherosclerosis) and patients with AS 2 and AS 3 in another group (moderate and severe atherosclerosis).

Laboratory analyses

MMP-8, 10, and TIMP-1 were assayed by specific enzyme-linked immunosorbent assay (Quantikine, R&D Systems, Abingdon, UK) according to the manufacturer's instructions with a serum dilution of 1:50, 1:1, and 1:100, respectively. The interassay coefficients of variation were $<8\%$ and detection limit for the assays were 0.31 ng/ml, 78.1 pg/ml, and 0.15 ng/ml.

Statistical analyses

Results are expressed as mean (s.d.). Univariate descriptive statistics were performed and differences among CKD types (I–III, IV–V without and with dialysis) and controls were tested using the analysis of variance test for comparisons between means of numerical variables, the Kruskal–Wallis test to compare the medians of numerical skewed variables, and the Pearson χ^2-test to compare frequency distributions of categorical variables (substituted by Fisher exact test when needed). We applied *post hoc* analyses (Bonferroni tests) for multiple paired comparisons among CKD types and controls. The AS classification (from AS 0 to AS 3) was used to study atherosclerosis burden and its related variables, once recoded into two groups to extract those with moderate or severe atherosclerosis (AS 2 and 3). In order to identify the impact of MMPs on the prediction of moderate or severe atherosclerosis, a multivariate logistic regression model was estimated. Dependent

variables were identified in the univariate analyses as those related to the presence of atherosclerosis (AS 2 or 3, $P<0.10$ in the univariate analyses) or those variables that might have a confounding effect. We included in the analyses: sex, age, stage of CKD (dialysis vs CKD stages I–III), previous CVD, type 2 diabetes mellitus, treatment with statins, cardiovascular risk score, C-reactive protein concentration, and MMP-8, 10, and TIMP-1, expressed as tertiles. We keep in the model only those characteristics with a statistically significant relationship (P-value <0.05) or a confounding effect over any of the coefficients of the rest of the variables.

DISCLOSURE

All the authors declared no competing interests.

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